

## MINIREVIEW

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## A Family Portrait of the RIO Kinases\*

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Nicole LaRonde-LeBlanc and Alexander Wlodawer<sup>1</sup>

From the Protein Structure Section, Macromolecular Crystallography Laboratory, NCI-Frederick, National Institutes of Health, Frederick, Maryland 21702-1201

The RIO family of atypical serine protein kinases has been first characterized only recently. It consists of enzymes that contain a unique domain with a characteristic kinase sequence motif and usually some additional domains. At least two RIO proteins, Rio1 and Rio2, are present in organisms varying from Archaea to humans, with a third Rio3 subfamily present only in multicellular eukaryotes. Yeast Rio1 and Rio2 proteins have been implicated in the processing of 20 S pre-rRNA and are necessary for survival of the cells. Crystal structures of *Archaeoglobus fulgidus* Rio1 and Rio2 have shown that whereas the overall fold of these enzymes resembles typical protein kinases, some of the structural domains, particularly those involved in peptide substrate binding, are not present. The mode of binding of nucleotides also differs from that found in typical protein kinases. Although it has been shown that both Rio1 and Rio2 have the enzymatic activity of kinases and are capable of autophosphorylation, the biological substrates of RIO proteins and their full biological role still remain to be discovered.

The RIO family of proteins was first characterized only 8 years ago, based on the studies of a *RIO1* (right open reading frame) gene, expressed constitutively at a low level in *Saccharomyces cerevisiae* (1). Subsequent detailed analyses of sequence data bases identified many genes that contain homologs of a kinase-like domain found in yeast Rio1 (2–4). A distinct Rio2 protein was subsequently identified in yeast (5). Although the RIO domain contains a kinase signature motif, it otherwise exhibits little sequence similarity with eukaryotic protein kinases (ePKs)<sup>2</sup> (3) and thus was classified as an atypical protein kinase (4). Representatives of the RIO family are present in organisms varying from Archaea to humans. All such organisms contain at least two such proteins, one that is more similar to yeast Rio1 and another with a slightly different RIO domain and a conserved N-terminal domain, homologous to yeast Rio2. Eventually, a third group named Rio3 was described, which is more similar to Rio1 but also contains a conserved N-terminal domain different than that of Rio2 (4). Members of the Rio3 subfamily have been found thus far only in multicellular eukaryotes. As shown in Fig. 1 each RIO subfamily is distinguished by specific sequence variations in the RIO kinase domains as well as by the presence of subfamily-specific, conserved N-terminal sequences. It was also reported that RIO kinases are present in a number of species of eubacteria with only a single representative per organism (2, 6). Examination of their sequences reveals that the bacterial RIO kinases are most similar to Rio1 in the N-terminal half and more similar to Rio2 in the C-terminal half of the kinase domain (Fig. 1). Thus it appears that the bacterial RIO kinases are related to both Rio1 and Rio2 enzymes and may represent the remnants of a common progenitor of the two subfamilies. This is interesting in view of the report that the KDO lipid kinases bear significant homology to the bacterial RIO kinases (6), and thus the RIO kinases may represent the evolutionary link between bacterial lipid kinases and ePKs.

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<sup>1</sup> To whom correspondence should be addressed: Bldg. 536, Rm. 5, MCL, NCI, Frederick, MD 21702-1201. Tel.: 301-846-5036; Fax: 301-846-6322; E-mail: wlodawer@ncifcrf.gov.

<sup>2</sup> The abbreviations used are: ePK, eukaryotic protein kinase; MBP, myelin basic protein; PKA, cAMP-dependent protein kinase; AMPPPNP, adenosine 5'-( $\beta$ , $\gamma$ -imino)triphosphate.

## RIO Kinases Play an Important Role in Ribosome Biogenesis

The founding member of the RIO family, Rio1 (sometimes also called Rrp10p for ribosomal RNA processing number 10 (7)), is an essential gene in *S. cerevisiae*, required for proper cell cycle progression and chromosome maintenance (3). In yeast cells deprived of Rio1, cell cycle arrest occurs in G<sub>1</sub> or mitosis, indicating that Rio1 activity is required for entry into S phase and exit from mitosis (3). In addition, both yeast Rio1 and Rio2 were identified as non-ribosomal factors necessary for late 18 S rRNA processing (5, 7). Depletion of Rio1 or Rio2 affects growth rate and results in an accumulation of 20 S rRNA (5), with depletion of Rio1 resulting in cell cycle arrest. Deletion of either Rio1 or Rio2 is lethal for the cells, suggesting that the two proteins perform distinct functions (7, 8). It has been demonstrated that the yeast RIO proteins are capable of serine phosphorylation *in vitro*, and conserved kinase catalytic residues are required for their *in vivo* function (5, 9). The cellular localization of Rio1 and Rio2 in yeast was investigated using green fluorescent protein-labeled proteins. Both Rio1 and Rio2 shuttle between nucleus and cytoplasm. The presence of green fluorescent protein-tagged Rio2 was detected in both compartments (9), but TAP-Rio2 was seen only in the nucleus (5). By contrast, Rio1 was seen solely in the cytoplasmic fraction (7, 9). The biological activities of the two proteins do not complement each other and they do not co-purify although both are associating with the same fractions that contain 20 S pre-rRNA (9). It was also shown that Rio2 is a component of the 43 S pre-ribosomal subunit (10).

## Expression and Biochemical Characterization of the RIO Proteins

Several RIO proteins have been expressed in *Escherichia coli*, and their enzymatic properties were characterized. Yeast Rio1 was cloned and expressed with a N-terminal His<sub>6</sub> tag (11). The protein was shown to be an active kinase, although the established substrates such as  $\alpha$ -casein, histones H1 and H2A, enolase, or myelin basic protein (MBP) were found to be phosphorylated very poorly. However, purified Rio1 showed strong ATP-dependent autophosphorylation, although the dephosphorylated enzyme was reported to be nearly inactive (11). *A. fulgidus* Rio1 was expressed in *E. coli* as a fusion protein containing the N-terminal His<sub>6</sub> tag followed by a tobacco etch virus protease cleavage site; the tag was subsequently removed during protein purification. The enzyme is capable of autophosphorylation on Ser-108 and can also phosphorylate MBP and histone H1. However, neither the absolute levels of enzymatic activity nor reaction kinetics of *A. fulgidus* Rio1 were investigated in detail. A mutant in which the putative catalytic residue Asp-196 was changed to alanine was only marginally active, whereas the S108A mutant that lacked the autophosphorylation site retained its enzymatic activity (12).

Yeast Rio2 was expressed in *E. coli* as a fusion peptide with glutathione S-transferase at its N terminus, and its activity was assayed in this form. The enzyme was capable of autophosphorylation in the presence of both Mg<sup>2+</sup> and Mn<sup>2+</sup> ions, although the latter ion was necessary for phosphorylation of MBP and increased the efficiency of autophosphorylation. A D229A mutant was shown to be marginally active, whereas the H227F mutant retained full activity (5). *A. fulgidus* Rio2 with its native N terminus was expressed in the same way as Rio1 from that organism (13), and it was also shown to be active in an autophosphorylation assay, although at a level much lower than that of Rio1. The level of autophosphorylation was similar in the presence of either Mg<sup>2+</sup> or Mn<sup>2+</sup> ions. The site of autophosphorylation was determined to be Ser-128. No mutants of this protein have been studied to date.

A Rio1 homolog from *Nicotiana tabacum* (NtRIO) has been expressed in *E. coli* as a cleavable glutathione S-transferase fusion protein (14). The full-length protein contained 562 amino acids, and its RIO domain (residues 144–380) exhibited 33% identity with *S. cerevisiae* Rio1. NtRIO is also capable of autophosphorylation, although the level of activity was 100-fold higher in the presence of the recombinant tobacco casein kinase CK2, either due to enhanced autophosphorylation or (more likely) through phosphorylation by CK2. The site(s) of autophosphorylation was not determined.

## Structural Characteristics of the RIO Domain

Determination of the crystal structures of *A. fulgidus* Rio2 and Rio1 made detailed structural characterization of each subfamily possible (12, 13). The overall fold of the kinase domain of these two enzymes is very similar (Fig. 2, A and B), although some significant local differences between the two proteins

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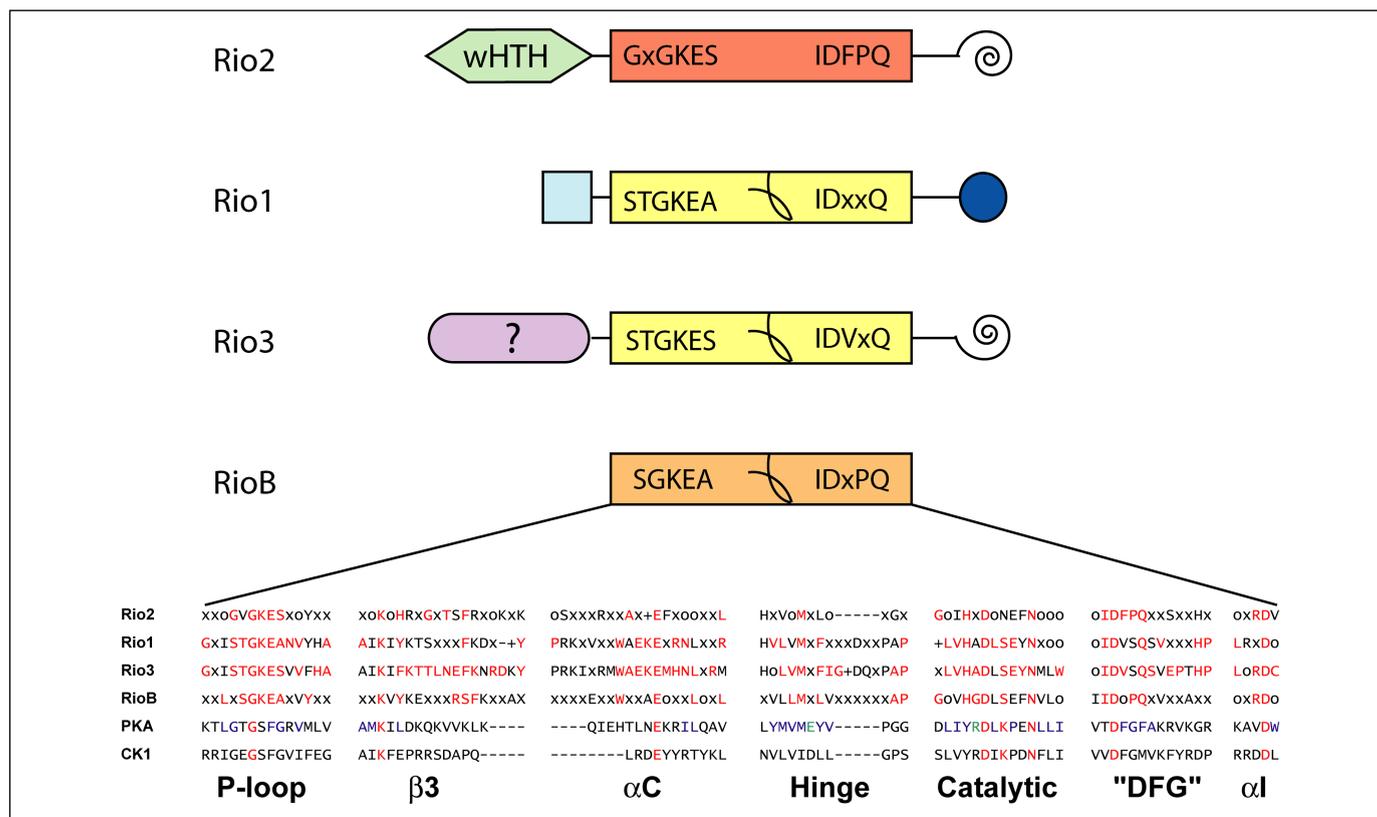


FIGURE 1. An outline of the primary structure of RIO kinases. Schematic diagram of the domain organization of each subfamily, showing sequence signatures within rectangles representing the RIO domains. Detailed sequences below show a comparison of conserved residues for the regions that define the membership in each subfamily. For the RIO proteins, residues identical among subfamily members are shown in red text. For PKA and CK1, residues that are conserved with the RIO proteins are also in red.

result in a root mean square deviation of 1.4 Å. As seen in both structures, the consensus RIO domain consists of an N-lobe comprised of a twisted β-sheet (β1–β6) and a long α-helix (αC) that closes the back of the ATP-binding pocket, a hinge region, and a C-lobe, which forms the platform for the metal-binding loop and the catalytic loop. However, the RIO kinase domain contains only three of the canonical ePK α-helices (αE, αF, and αI) in the C-lobe. In both Rio1 and Rio2, an additional α-helix (αR) located N-terminal to the canonical N-lobe β-sheet extends the RIO domain (Fig. 2, A and B). Unlike ePKs, all RIO domains contain an insertion of 18–27 amino acids between αC and β3. That region was named the “flexible loop” because it was largely disordered in the structure of Rio2. An equivalent chain was partially disordered in the structure of Rio1 complexed with ATP but was generally ordered, although with some side chains not visible in the enzyme without ATP. Comparison of the RIO structures with that of cAMP-dependent protein kinase (PKA) showed that the RIO kinase domains lack the activation or “APE” loop (subdomain VIII), as well as subdomains X and XI of canonical ePKs. In addition to the N-terminal α-helix specific to the RIO domain, Rio1 contains another two α-helices N-terminal to the RIO domain, as opposed to the complete winged helix domain present in Rio2.

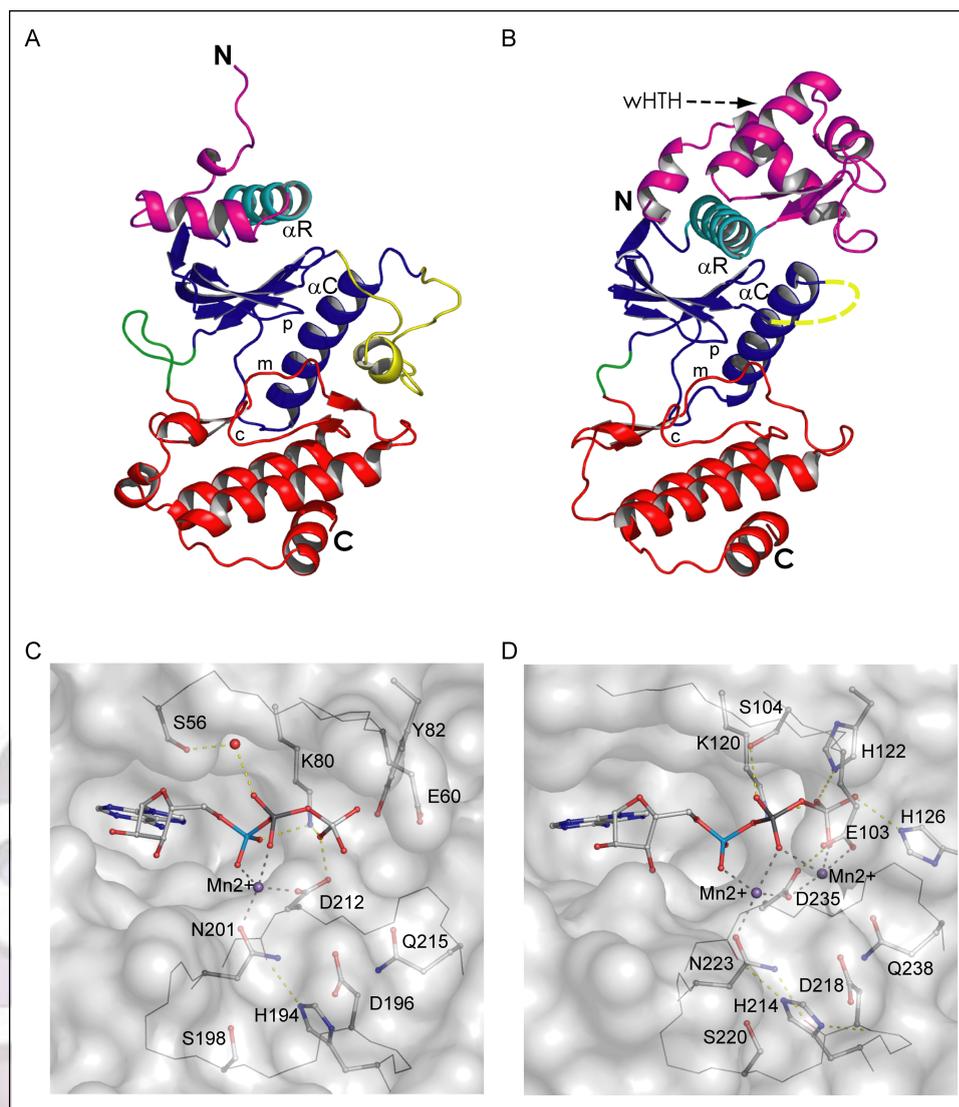
### Nucleotide Binding to RIO Kinases

The structures of Rio1 and Rio2 have been solved in complexes with ATP, ADP, and AMPPNP. The mode of binding of the nucleotides is similar in the two subfamilies, although some differences exist. The nucleotides are bound between the N-lobe and the C-lobe and are in contact with the hinge region, the P-loop, the metal-binding loop, and the catalytic loop (Fig. 2). In Rio1, the adenosine base participates in two hydrogen bonds with the hinge region, one from the peptide carbonyl oxygen of conserved Glu-148 to the amino group N6 and one from the peptide amine of Ile-150 to the indole nitrogen N1. The ribose moiety is contacted through water-mediated hydrogen bonds from the 2'-hydroxyl to Glu-162 and 3'-hydroxyl to the backbone carbonyl oxygen of conserved Tyr-200. The triphosphate group is held in place by several contacts with conserved residues (Fig. 2C). The P-loop interacts through three water-mediated hydrogen bonds: between the hydroxyl side chain of conserved Ser-56 and one of the β-phosphate oxygens, between the backbone amine of

conserved Lys-59 to the oxygen bridging the β- and γ-phosphate, and between the side chain carboxylate of conserved Glu-81 to one of the γ-phosphate oxygens. The single Mn<sup>2+</sup> ion seen in the ATP complex coordinates oxygens from the β- and α-phosphates, the carbonyl oxygen of the catalytic loop residue Asn-201, and a carboxyl oxygen from the metal-binding loop residue Asp-212 along with two water molecules. Additional contacts with the triphosphates are made through the side chain amino group of Lys-80 (conserved in all protein kinases) to α- and γ-phosphate oxygens, through a direct hydrogen bond between a carboxyl oxygen of the side chain of Asp-212 and a γ-phosphate oxygen, and interestingly, through a water-mediated interaction between the indole nitrogen of Trp-116 from the end of helix αC and a γ-phosphate oxygen. In the ADP complex, a water molecule replaces the γ-phosphate, but no significant conformational changes are observed in the active site.

Although the adenosine ring is buried deeper in Rio1 than in Rio2 proteins, the γ-phosphate is significantly more accessible in the former (Fig. 2D). In the structure of Rio2 bound to ATP and Mn<sup>2+</sup>, the γ-phosphate is buried through the ordering and binding of three residues of the N-terminal end of the flexible loop (15). The P-loops of Rio1 and Rio2 are in different positions relative to the γ-phosphate, closer in the latter than in the former. The γ-phosphate is also more tightly bound in Rio2, where a second metal ion is seen which coordinates the γ-phosphate, and each γ-phosphate oxygen participates in two interactions with the protein. In the case of Rio1, no metal ion is seen contacting the γ-phosphate, and one of the phosphate oxygens makes no interactions with the protein. It is therefore conceivable that release of the γ-phosphate may be more difficult in Rio2 than Rio1 or may require further rearrangement of the Rio2 molecule. The absence of a metal ion contact between the γ-phosphate and the metal ion in the Rio1 complex is curious since the metal ion is required for catalysis of phosphoryl transfer. Therefore, it is conceivable that in the presence of substrate a second metal ion binding site may be occupied to participate in the enzymatic reaction.

The conformation of the nucleotides in the RIO kinases is unique when compared with ePKs, such as serine/threonine kinases PKA and CK1 (casein kinase) or the insulin receptor tyrosine kinase IRK (12, 15–18). The difference in position of the γ-phosphate results in a difference in the distance between



**FIGURE 2. Three-dimensional structures of RIO kinases.** *A*, *A. fulgidus* Rio1. *B*, *A. fulgidus* Rio2. N-lobes of the kinase domain are colored *blue*, C-lobes *red*, and hinge regions *green*. Helix  $\alpha$ R that forms part of the RIO kinase domain, but is not found in ePKs, is colored *cyan*, and the winged helix domain present only in Rio2 and the additional helices present in Rio1 are colored *magenta*. The flexible loop is *yellow* and is represented by a *dashed line* in *B*, since it was not ordered in the crystal structure of Rio2. The catalytic loop, the metal-binding loop, and the P-loop are labeled as *c*, *m*, and *p*, respectively. Bound ATP is shown in the active site of Rio1 (*C*) and the active site of Rio2 (*D*). Catalytic residues and those which contact the triphosphate group and the metal ion(s) are shown in *stick* representation and are labeled.

the catalytic aspartate residue and the  $\gamma$ -phosphate. In PKA, this distance is 3.8 Å, whereas in Rio2 an equivalent distance is 5.8 Å and in Rio1, 5.1 Å. It should be noted that in IRK this distance is also 5.8 Å for a complex with AMPPNP. Combined, these data suggest that the detailed mechanism by which the catalytic aspartate of RIO kinases participates in phosphoryl transfer may not be identical to that employed in known serine/threonine ePKs. It has been shown that mutation of the putative catalytic aspartate to alanine in phosphorylase kinase results in an enzyme that still retains about 0.1% of its catalytic efficiency (19). Whereas no detailed kinetic data have been collected for RIO proteins, equivalent mutants of yeast Rio2 and *A. fulgidus* Rio1 retain a low level of activity (5, 12).

### Additional Domains of RIO Kinases

Analysis of the sequence alignments of the RIO proteins shows the presence of additional domains specific for each subfamily (Fig. 1). Only one such domain has been characterized structurally to date. The N-terminal domain of Rio2, conserved in different members of this subfamily and not present in the Rio1 subfamily, is structurally homologous to the winged helix (wHTH) domain (Fig. 2*B*). In AfRio2, the N-terminal domain contains four  $\alpha$ -helices followed by two  $\beta$ -strands and a fifth  $\alpha$ -helix. A strand that connects the second and third  $\alpha$ -helices combines with the other two  $\beta$ -strands to form a  $\beta$ -sheet, and the loop between the second and third  $\beta$ -strands is called a "wing," which gives the wHTH its name. No analogous combination of a wHTH domain and a kinase domain has been previously reported.

The nearest structural neighbors to the AfRio2 wHTH domain include transcription factors MarR and SlyA and histone linker protein GH5. The most

commonly reported function of such domains is DNA binding, and they have been shown to bind DNA in two different ways (20, 21). The most common DNA-binding mode, first reported in the crystal structure of transcription factor HNF-3 bound to DNA, is through site-specific interactions in the major grooves with residues on  $\alpha$ -helix 3 (22). The second mode, characterized by the crystal structure of hREFX1 bound to DNA, involves specific interactions of W1,  $\beta$ 2, and  $\beta$ 3 with the major groove of the DNA (23). Initial experiments have indicated that Rio2 may bind nonspecifically to single-stranded oligonucleotides.<sup>3</sup>

The structure and function of other domains that are part of the RIO proteins are not known at this time. They include a lysine-rich domain at the C terminus of the Rio1 proteins from yeast to humans and a N-terminal domain for which no homology was detected in the Rio3 subfamily. In yeast, deletion of the last 80 residues of the C-terminal domain results in loss of Rio1 function with an increase in *in vitro* kinase activity, suggesting a separate function for this domain (3).

### Human RIO Kinases

At this time, no reports of purification of human RIO kinases can be found, but some of these enzymes have been characterized at the DNA/RNA level. The levels of Rio1 mRNA expressed in human tumor tissues were investigated, indicating that this gene is overexpressed in comparison with the adjacent non-cancerous tissue and exhibits differential tissue distribution (24). The

<sup>3</sup> N. LaRonde-LeBlanc and A. Wlodawer, unpublished data.

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principal transcript of its gene, designated AD034 (NM\_031480), is a protein consisting of 569 amino acids. A splice variant that encodes a truncated protein consisting of the C-terminal 328 amino acids (NM\_153005) was also reported. The DNA sequence of human Rio2 (NM\_018343), also designated FLJ11159, encodes a protein consisting of 553 amino acids. Human Rio3 was also characterized at the DNA level only, in two splice variants, and was identified as a protein up-regulated in the core of malignant melanomas (25, 26). The longer variant (NM\_003831) consists of 520 amino acids and was characterized to be a homolog of *Aspergillus nidulans* sudD (25). The shorter variant (NM\_145906) contains the N-terminal 425 amino acids only. It is not clear if the shorter variants of Rio1 and Rio3 are ever translated into proteins. No mutations in any of the human RIO proteins were found in a screen of the complete protein kinase gene family aimed at identifying somatic mutations in human breast cancer (27). This result might be interpreted as indicating that functional RIO proteins are necessary for survival of the cells in humans, as they are in yeast.

### Conclusions

The ubiquitous presence of RIO kinases in a variety of organisms, the requirement that the enzymatic activity be present for cell survival, and the involvement in ribosome biogenesis, one of the fundamental processes in all living cells, all suggest that this family of atypical kinases should be further investigated with the aim of establishing if it might present a novel target for drug design. The difficulty in designing specific kinase inhibitors lies in the similarity of their target sites, which usually are limited to the nucleotide-binding regions. Although considerable progress in creating very specific inhibitors of ePKs has been recently reported (28), the advantage of atypical kinases such as RIOs might be in their differences from the better known counterparts. The lack of the peptide substrate binding loop in RIO kinases, for example, suggests a potentially very different binding mode of the protein substrates. No structural data on peptide binding are available at this time, but such information should become available if the present rapid pace of investigations of this novel kinase family continues into the future.

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